

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1571875> since 2017-06-01T11:59:41Z

Published version:

DOI:10.1111/liv.12807

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

Saitta, Carlo; Tripodi, Gianluca; Barbera, Adalberto; Bertuccio, Antonio; Smedile, Antonina; Ciano, Alessia; Raffa, Giuseppina; Sangiovanni, Angelo; Navarra, Giuseppe; Raimondo, Giovanni; Pollicino, Teresa. Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma. LIVER INTERNATIONAL. 35 (10) pp: 2311-2317.
DOI: 10.1111/liv.12807

The publisher's version is available at:

<http://doi.wiley.com/10.1111/liv.12807>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/2318/1571875>

Hepatitis B virus (HBV) DNA integration in patients with occult HBV infection and hepatocellular carcinoma

Saitta C, Tripodi G, Barbera A, Bertuccio A, Smedile A, Ciano A, Raffa G, Sangiovanni A, Navarra G, Raimondo G, Pollicino T

Abstract

Background & Aims

Hepatitis B virus (HBV) DNA integration in the host genome is a major mechanism responsible for the etiopathogenetic role exerted by HBV in hepatocellular carcinoma (HCC) development. Extensive analyses evaluating viral integration in HBV surface antigen (HBsAg) negative patients with occult HBV infection (OBI) have not yet been performed. The aim of this study was to investigate and characterize HBV DNA integration in HCC tissues from OBI patients.

Methods

Tumour DNA extracts from 69 HCC patients (49 HBsAg-negative with occult infection diagnosed by HBV DNA detection in tumour tissues; 10 HBsAg-positive and 10 HBsAg-negative/OBI-negative as control groups) were examined by Alu-PCR technique to reveal HBV DNA integration into the host genome. The molecular characterization of the virus-genome junctions was performed by cloning and sequencing analyses.

Results

Integrated HBV DNA was detected in 37/49 (75.5%) OBI-positive HCC samples, in 8/10 (80%) HBsAg-positive and in 0/10 OBI-negative HCC samples. Nine of 37 (24.3%) integrated viral sequences from OBI-positive cases were inside human genome coding regions and in the remaining cases the localization at intergenic level was frequently adjacent to coding genes. Concerning viral integrants in OBI cases, X gene sequences were found in 14 cases, preS/S sequences in 13, Core sequences in 7, and Polymerase gene sequences in three cases.

Conclusions

In analogy to what occurs in HBsAg-positive cases, HBV DNA integration is highly prevalent in OBI-related HCCs, it mainly involves X and preS/S viral genomic regions and it frequently occurs at the level of regulatory and functional genes.

Abbreviations

ADCY5 adenylate cyclase 5

BCP basal core promoter

CNKS3 CNKS family member 3

DNTTIP1 deoxynucleotidyltransferase, terminal, interacting protein 1

EXOC4 exocyst complex component 4

FBXL17 F-box and leucine-rich repeat protein 17

GFOD1 glucose-fructose oxidoreductase domain-containing 1

GNAO1 guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O

HBsAg HBV surface antigen

HBV hepatitis B virus

HCC hepatocellular carcinoma

OBI occult HBV infection

PCR polymerase chain reaction
PI4K2A phosphatidylinositol 4-kinase type 2 alpha
PKHD1 polycystic kidney and hepatic disease 1
SCARB1 scavenger receptor class B, member 1
TMEM107 transmembrane protein 107
TSC22D1 TSC22 domain family, member 1
UDG uracil DNA glycosylase
ZNF561 zinc finger protein 561

Hepatocellular carcinoma (HCC) is a common solid tumour worldwide and the third leading cause of cancer mortality [1]. Hepatitis B virus (HBV) is generally considered a main etiologic cause of HCC and it appears to be involved in many of the complex and multifactorial pathogenetic mechanisms underlying HCC development [2]. In fact, HBV infection may exert its pro-oncogenic role both indirectly – by inducing a chronic state of necroinflammatory liver injury that may progress through cirrhosis to HCC – and directly by the synthesis of viral proteins provided with pro-oncogenic properties and above all by the propensity of its DNA to integrate into the host's genome, an event inducing a wide range of genetic alterations and capable by itself of determining hepatocyte transformation [3]. Much evidence suggests that HBV may maintain its tumorigenic properties even in cases of occult infection that is defined as the long-lasting persistence of HBV DNA in the liver – and in some cases also in the serum – of HBV surface antigen (HBsAg) negative individuals [4]. Actually, in spite of the strong suppression of HBV replication and gene expression typical of the occult HBV infection (OBI), numerous data from both human patients and animal models have demonstrated that free episomal viral genomes may persist in tumour livers from HCC individuals with occult viral infection, maintaining very low but quantifiable replication and transcription activities [5, 6]. Moreover, studies performed since the early '80s have shown HBV DNA integration in HCC samples from HBsAg negative patients [7, 8]. However, these studies were mainly performed by the use of the Southern blot technique, a methodological approach with several limitations in sensitivity and that requires a quite large amount of DNA extracts for viral integrant detection. Recently, more sensitive approaches have been utilized for investigating HBV integration. In particular, the Alu-polymerase chain reaction (PCR) technique was applied to examine HCC livers from HBsAg-negative subjects, confirming the possible presence of viral integrates in these cases [9, 10]. However, evaluation of HBV DNA integration in tumours from HBsAg-negative individuals has been performed in a very limited number of cases so far. The aims of the present study were to evaluate the prevalence of HBV DNA integration in tumour DNA extracts from HBsAg-negative/HBV DNA positive patients and to characterize integrated viral DNA and host genome sites of integration in these patients.

Patients and Methods

Liver tumour specimens from 80 HBsAg-negative cirrhotic patients with HCC [57 men and 23 women; mean age, 64.9 (± 10.4 years)], randomly collected from January 2006 to December 2008 at three Italian liver centres located in distinct geographic areas of the country, were studied. Forty-five of the 80 patients were HCV infected and 35 were anti-HCV-negative individuals, eight of whom had diagnosis of alcoholic liver disease, eight of non-alcoholic steatohepatitis and 19 had cryptogenic liver disease. All patients had preserved liver function and belonged to Child-Pugh class A. Forty-one patients were anti-HBc positive and 13 of them were also antibody to HBsAg positive, whereas 39 cases were negative for all HBV serum markers. Paired non tumour specimens were available from 20 patients (14 men and six women, 12 HCV infected, two affected by alcoholic liver disease, two by non alcoholic steatohepatitis, and four by cryptogenic liver disease). Tissue samples had been obtained by surgical resection in 71 cases and by percutaneous needle biopsy in nine cases and immediately frozen and stored at -80°C .

All frozen tumour specimens were tested for occult HBV DNA through previously described methods based on nested PCR amplification [6]. Briefly, DNA was extracted from each specimen by the use of MasterPureTM DNA Purification Kit (epicentre, Madison, WI, USA) and examined for the presence of HBV genomes by performing four different in-house nested-PCR amplification assays to detect preS-S, preCore-

Core, Polymerase and X HBV genomic regions, respectively. Appropriate negative and positive controls were included in each PCR experiment. In particular, as negative controls we included in each test (i) liver tissue DNA extracts from samples known to be negative for HBV DNA; (ii) DNA-free reaction buffer; (iii) water. In addition, to eliminate false negative results, beta-globin was used as a house-keeping gene. Moreover, direct sequencing of all amplicons confirmed the specificity of the reactions.

Canonically, an occult HBV infection is defined as the intrahepatic long-lasting persistence of episomal viral DNA and recognized through detection of at least two different viral genomic regions at nested-PCR analysis [11]. Since this study was focused on investigation of viral integrants and not of free genomes in tumour cells, we assayed the presence of integrants in all the liver DNA extracts testing positive for HBV sequences, including those reacting positive for only one of the four different HBV genomic regions analysed. In addition, we performed the same analysis on tumour tissues from two different HCC control groups, 10 HBsAg-positive subjects (seven males, mean age 64.1 ± 10.4 years; and 10 HBsAg-negative/OBI-negative individuals (nine males, mean age 68.1 ± 11.2 years). Paired non tumour tissues were available from 2 HBsAg positive cases and were also analyzed. HBV DNA insertion into the hepatocyte genome was investigated by applying the Alu-PCR technique in accordance to described methods [12, 13]. Briefly, amplification was carried out in a final volume of 50 μ l, containing 100 ng of genomic DNA as a template, 10 pM of Alu primer and 100 pM of HBV primers designed for three distinct viral genomic regions (Core, X and preS/S). A hot start technique was used and one unit of uracil DNA glycosylase (UDG) was added to each tube after the first 10 cycles of amplification; the tubes were incubated for 30 minutes at 37°C and heated for ten minutes at 94°C to break the DNA strands at apurinic dUTP sites. A 'touchdown' PCR technique was then employed for a total of 40 cycles. Five microlitre of the amplified products were subjected to nested PCR with internal primers (Table 1). In order to confirm the specificity and increase the sensitivity of the PCR results, Southern Blot analysis was performed on all the amplified products following standardized procedures. Nucleotide sequences of the PCR products containing viral-host junctions were determined by direct sequencing and the use of the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequencing products were resolved in an automatic DNA sequencer (ABI PRISM 3500 Dx Genetic Analyzer; Applied Biosystems). Samples that tested negative after PCR amplification on agarose gel, but subsequently tested positive for HBV DNA integration by Southern Blot analysis were cloned into the PCR-TOPO vector (Invitrogen, Milan, Italy). Single clones were selected for sequencing, as described above. Nucleotide sequences were assessed using the BLAST search system. HBV integrated sequences were then better characterized using the CLUSTAL W program.

Table 1. Sequences of the primers used for Alu-PCR

| Primer | Primer sequence (5'-3') | HBV position |
|---|---|--------------|
| 1. Nucleotide positions of the primers are numbered from the only Eco RI site and the nomenclature is according to Galibert <i>et al.</i> ([14].) | | |
| Alu-sense | CAGUGCCAAGUGUUUGCUGACGCCAAAGUGCUGGGAUUA | |
| Alu-antisense | AUUAAACCCUCACUAAAGCCUCGAUAGAUYYRCCAYUGCAC | |
| Alu-sense (tag) | CAAGTGTTTGCTGACGCCAAAG | |
| Alu-antisense (tag) | ATTAACCCCTCACTAAAGCCTCG | |
| HBV 1 (X) | ACAUGAACCUUUACCCGUUGC | 1.133–1.154 |
| HBV 2 (X) | TGCCAAGTGTTTGCTGACGC | 1.174–1.193 |
| HBV 3 (X) | CTGCCGATCCATACTGCGGAAC | 1.258–1.279 |
| HBV 1 (Core) | GAGUUCUUCUUCUAGGGGACCUG | 2.387–2.365 |
| HBV 2 (Core) | AGTGCGAATCCACACTC | 2.284–2.268 |
| HBV 3 (Core) | GGAAGGAAAGAAGTCAGAAGG | 1.978–1.958 |
| HBV 1 (preS) | AUCUCAAUCCGCCGUCGCA | 2.407–2.426 |
| HBV 2 (preS) | AGAAGATCTCAATCTCGGG | 2.426–2.444 |
| HBV 3 (preS) | GTTAGTATTCCTTGGAATCATAAGGT | 2.454–2.479 |

| Primer | Primer sequence (5'-3') | HBV position |
|-----------|-------------------------|--------------|
| HBV 1 (S) | GGUCACCAUAUUCUUGGGAA | 2.821–2.840 |
| HBV 2 (S) | CCTCTGGGATTCTTTCCC | 2.914–2.931 |
| HBV 3 (S) | AATCCAGATTGGGACTTCAA | 2.971–2.990 |

Data were statistically analysed by means of χ^2 test for categorical data, and by means of Student *t*-test for continuous data. $P < 0.05$ (two tailed) was considered significant. The study protocol was approved by the ethics committee of the 'Azienda Ospedaliera Universitaria Policlinico G.Martino' of Messina, performed according to the principles of the Declaration of Helsinki, and written informed consent was obtained from all patients.

Results

Forty-nine of the 80 tumour samples (61.2%) from HBsAg-negative patients with HCC tested positive for HBV DNA. These 49 did not show any statistical difference with the 31 HBV DNA negative cases in terms of age, sex, anti-HBc status and etiology of the liver disease (Table 2). In particular, 10 cases were positive for all four HBV genomic regions examined (S, Core, Polymerase, X), 15 were positive for three genomic regions, 14 for two genomic regions and 10 for one, respectively (Table 3). Tumour DNA extracts from all the 49 cases were analysed by Alu-PCR technique to reveal HBV DNA integration into the host genome. The same analysis was performed on tumour samples from 10 randomly selected HBsAg-negative/OBI-negative and 10 HBsAg-positive individuals with HCC included as controls.

Table 2. Demographical, clinical and serologic characteristics of 80 HCC patients according to the hepatitis B virus (HBV) DNA positive or negative status

| | HBsAg neg/HBV DNA pos (n.49) | HBsAg neg/HBV DNA neg (n.31) | P |
|--|------------------------------|------------------------------|------|
| Sex (M/F) | 35/14 | 22/9 | n.s. |
| Mean age at HCC diagnosis (\pm SD) | 64.3 (\pm 9.1) | 65.2 (\pm 10.4) | n.s. |
| Etiology of liver disease (HCV/alcohol/NASH/cryptogenic) | 27/7/8/7 | 18/5/4/4 | n.s. |
| Anti-HBV antibodies (positive/negative) | 28/21 | 13/18 | n.s. |

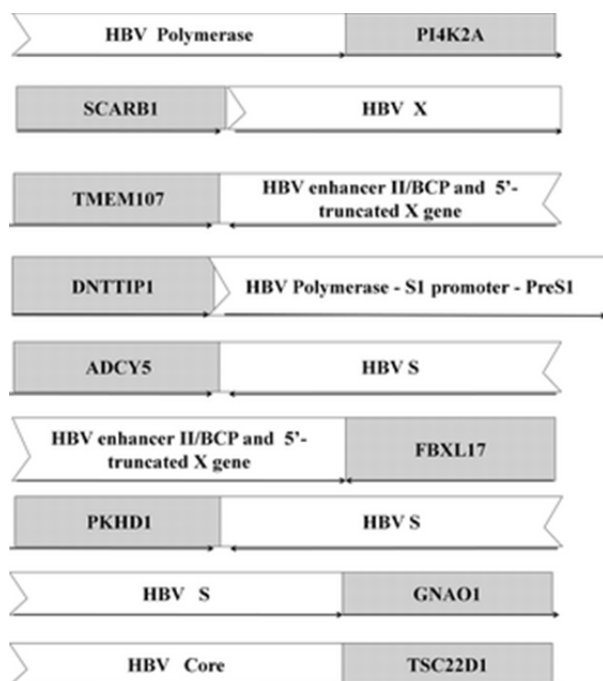
Table 3. Hepatitis B virus genomic regions testing positive in 49 tumour tissues analysed by four different nested PCR amplifications

| | Polymerase | PreCore/Core | PreS/S | X |
|-------------------------------------|------------|--------------|----------|----------|
| 1. n.d., not detected. | | | | |
| No. cases positive in four regions | | | | |
| 10 | Positive | Positive | Positive | Positive |
| No. cases positive in three regions | | | | |
| Four cases | Positive | Positive | Positive | n.d. |
| Four cases | Positive | Positive | n.d. | Positive |
| Four cases | n.d. | Positive | Positive | Positive |
| Three cases | Positive | n.d. | Positive | Positive |
| No. cases positive in two regions | | | | |
| Four cases | n.d. | n.d. | Positive | Positive |
| Three cases | Positive | n.d. | n.d. | Positive |
| Three cases | Positive | n.d. | Positive | n.d. |
| Two cases | n.d. | Positive | Positive | n.d. |
| Two cases | n.d. | Positive | n.d. | Positive |
| No. cases positive in 1 region | | | | |
| Six cases | n.d. | n.d. | n.d. | Positive |
| Three cases | n.d. | n.d. | Positive | n.d. |
| One case | Positive | n.d. | n.d. | n.d. |

Integrated HBV DNA was detected in 37/49 (75.5%) HBsAg–negative/OBI–positive HCC samples, in 8/10 (80%) HBsAg–positive tumours and in none of the HBsAg–negative/OBI–negative neoplastic specimens. In the OBI positive group, the occurrence of viral integration was independent of the etiology of the liver disease including HCV infection. In the OBI positive tissues, viral integration was randomly distributed throughout the host genome. In fact, chromosome 1 was the target of HBV integration in four cases, chromosome 6 in three cases, each of the chromosomes 3, 7, 12, 15, 16, 17 and 20 in two cases, and each of the chromosomes 5, 8, 10, 13 and 14 in one case. Twelve cellular flanking sequences were repetitive or unidentified sequences, thus not allowing the precise localization of the genomic site of integration.

Nine out of 37 integrated viral sequences (24.3%) were revealed inside coding regions of human genome (*intragenic* integrations) (Fig. 1). In particular: (i) phosphatidylinositol 4-kinase type 2 alpha (*PI4K2A*), on chromosome 10; (ii) scavenger receptor class B, member 1 (*SCARB1*), on chromosome 12; (iii) transmembrane protein 107 (*TMEM107*), on chromosome 17; (iv) deoxynucleotidyltransferase, terminal, interacting protein 1 (*DNTTIP1*), on chromosome 20; (v) adenylate cyclase 5 (*ADCY5*), on chromosome 3; (vi) F-box and leucine-rich repeat protein 17 (*FBXL17*), on chromosome 5; (vii) polycystic kidney and hepatic disease 1 (*PKHD1*), on chromosome 6; (viii) guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O (*GNAO1*), on chromosome 16; (ix) TSC22 domain family, member 1 (*TSC22D1*), on chromosome 13. Notably, most of these genes are importantly involved in angiogenesis, cell growth regulation, adhesion and cell signalling, and have been reported to be potentially implied in carcinogenic processes [15–22]. Seventeen out of 37 viral integrants were found at variable distances from genomic coding sequences (*intergenic* integrations) and six of them included viral regulatory elements. Of note, nine integrants were located closely (less than 34 Kb, range 56 base pairs – 33 Kb) to coding regions, thus at a distance at which the viral integrants might cause a deregulation of the gene expression [23]. Orientation of HBV genomic sequences was either the same or opposite to the cellular gene orientation.

Figure 1.



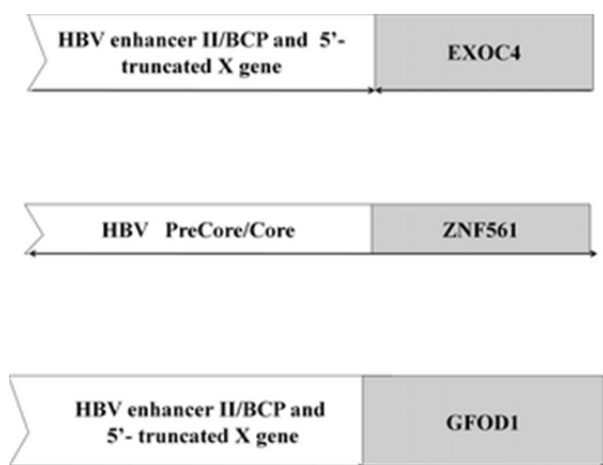
Hepatitis B virus (HBV) DNA *intragenic* integrations detected in tumour samples from HBsAg-negative patients. Dotted box: genomic coding sequence. Open box: HBV sequence. Bold arrow: ORF orientation. BCP: basal core promoter. *PI4K2A*: phosphatidylinositol 4-kinase type 2 alpha. *SCARB1*: scavenger receptor class B, member 1. *TMEM107*: transmembrane protein 107. *DNTTIP1*: deoxynucleotidyltransferase, terminal, interacting protein 1. *ADCY5*: adenylate cyclase 5. *FBXL17*: F-box and leucine-rich repeat protein 17. *PKHD1*: polycystic kidney and hepatic disease 1. *GNAO1*: guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O. *TSC22D1*: TSC22 domain family, member 1.

Concerning the viral integrants, X gene sequences were found in 14 cases [double site of X integration in one case; three X integrants included the enhancer II region and basal core promoter (BCP) and two the enhancer I region]; S gene sequences in 11 cases; Core gene sequences in seven cases (one integrant included the BCP); Polymerase gene sequences in three cases; the PreS1/PreS2 region including the preS/S promoter was revealed in one case; and the Polymerase region – also comprising S1 promoter and PreS1 region – was found in one case.

Hepatitis B virus sequences were detected in 12 of the 20 paired non tumour tissues analyzed, and six out of these 12 tissues had HBV integrants (five cases had integration also in the corresponding tumours whereas one case tested negative in the corresponding tumour specimen). The viral integrants were located at intergenic level of the human genome and far from coding genes in all these six cases. Moreover, the sites of integration were different from those revealed in the corresponding tumour tissues when present. Integrants corresponded to X gene sequences in three cases, to preS/S sequences in two cases and to Core sequences in one case.

Integrants were found inside repetitive sequences of human genome in 5 out of the 8 HBsAg positive cases. In the other three cases, integrants were detected at *intragenic* level (Fig. 2). In particular, the genes targeted by the integrational events were (i) exocyst complex component 4 (*EXOC4*), on chromosome 7, in one case, (ii) zinc finger protein 561 (*ZNF561*), on chromosome 19, in one case and (iii) glucose-fructose oxidoreductase domain-containing 1 (*GFOD1*), on chromosome 6, in the remaining case. Notably, in the last case an additional site of integration was revealed with an *intergenic* integration found 45 Kb far from the starting codon of CNKSR family member 3 (*CNKSR3*), on chromosome 6. Overall, the viral integrants revealed in the HBsAg-positive group were X gene sequences in four cases (two of which included the enhancer II region/BCP), S gene sequences in three cases and Core gene sequences in two cases (one of which included the BCP region). Paired non tumour tissues from 2 HBsAg positive cases (both positive for integration in the corresponding HCC specimens) were analyzed, and only one showed the presence of HBV integration (preS/S gene sequences) that was located far from coding regions and at different genomic level compared to that detected in the corresponding tumour tissue.

Figure 2.



Hepatitis B virus (HBV) DNA *intragenic* integrations detected in tumour samples from HBsAg-positive patients. Dotted box: genomic coding sequence. Open box: HBV sequence. Bold arrow: ORF orientation. BCP: basal core promoter. *EXOC4*: exocyst complex component 4. *ZNF561*: zinc finger protein 561. *GFOD1*: glucose-fructose oxidoreductase domain containing 1.

Discussion

We found integrated HBV genomic sequences in 75% of HBV DNA positive tumour tissues from HBsAg–negative patients. This very high prevalence was almost identical to that found in tumours from a control

group of HBsAg-positive individuals, and comparable to literature data reporting HBV DNA integration in over 80% of HCC from patients with overt infection [3, 23].

Indeed, HBV integration into the hepatocyte genome is an event occurring early in the course of the infection, preceding the development of HCC and largely recognized as a major factor implicated in the HBV-induced hepatocarcinogenesis [24-28]. In this context, studies conducted since the early '80s in HBsAg negative individuals without HCC revealed the presence of HBV DNA integration in all the possible stages of liver disease [7, 29], although prospective studies evaluating the possible association with HCC development over time have not been performed so far. The integration does not involve the full-length viral DNA but subgenomic portions of it, and it has no role in the HBV life cycle. Consequently, one may assume that the transforming effects of the integration may be exerted independently of the contemporary presence of active viral replication. This consideration is a key point enhancing the importance of our findings that show a very high prevalence of viral integrants in HCC patients with OBI that is typically characterized by the absence – or very low levels – of active viral replication [30]. HBV integration plays a complex role in HCC development. It may have (i) insertional mutagenic effect and may induce genetic instability [31]; (ii) it may modify gene expression (*cis-activation*) or alter structure and function of the produced cellular proteins when integration occurs into cellular genomic regulatory regions or coding regions [32]; (iii) it may produce mutated viral proteins such as truncated X or preS/S proteins which may activate signalling pathways implicated in tumorigenesis (*trans-activation*) [33].

Literature data concerning HBsAg-positive liver cancers largely show that HBV integration into the hepatocyte genome apparently occurs at random since integrants can be detected at multiple, different sites of various chromosomes [34]. However, when a large-scale analysis of the sites of viral insertions was performed, a quite high frequency of integration emerged at the level of or near to cellular regulatory genes involved in the cell life cycle (cell signalling, growth control, proliferation, viability, etc.) [10, 23, 35]. In analogy, in our tumours from OBI-positive patients the viral integration was distributed in different sites of various chromosomes but in a considerable proportion of them they were located within or near regulatory genes, including genes involved in cell growth and adhesion, angiogenesis and cell signalling. Another important aspect concerns the analysis of HBV integrants. It is known that the integrated viral sequences differ from each other in size and structure in HBsAg positive HCC. Moreover, most cases show the integration of subgenomic fragments corresponding to the X or to the preS/S regions with frequent rearrangements, and the inserted sequences often include viral regulatory elements that may exert their function on human genes located even many kilo-bases far from the site of insertion [23]. Similarly, in our OBI patients we found that the integrated viral sequences correspond to X or preS/S genomic regions in the vast majority of cases, and could be rearranged, truncated and comprehensive of viral regulatory elements. Summarizing, our data show that HBV integration is absolutely comparable between HBsAg-positive and OBI-positive HCC in terms of frequency of the event, sites of insertion into the host's genome and viral DNA integrates. HBV-related HCC may develop in livers with mild or even absent disease (and OBI has been found to be involved in similar cases occurring in HBsAg-negative cancer patients) [13]. However, in the large majority of HBsAg patients the HCC develops on cirrhotic livers, thus suggesting that also in cases with overt HBV infection the multifactorial processes involved in hepatocyte transformation and neoplasm growth usually also require – together with the direct pro-oncogenic mechanisms – the indirect mechanism represented by the long-lasting necro-inflammation induced by the active viral infection. Actually, all our OBI-positive patients had cirrhosis related to HCV infection in most cases and to steatohepatitis, alcohol or unknown etiology in the others, and we are tempted to speculate that, in the combination of factors playing roles in HCC development, the cause of liver disease may have been responsible for the chronic hepatitis and cirrhosis while the persisting intrahepatic HBV DNA, and mainly its integrated forms, may have provided important and direct tumorigenic effects. In this context, we should consider that although cirrhosis is a very important pre-neoplastic condition, it is not *per se* sufficient to induce HCC, since only some of the cirrhotic patients will develop HCC. On the other hand, clear differences exist among cirrhotics with HCC in terms of time of liver cancer occurrence, since it may develop early after cirrhosis establishment or many years after this event. In our very recent observational cohort study evaluating the clinical evolution of chronic hepatitis C patients according to their OBI status, we showed that OBI was

strongly associated with HCC development [36]. In the present study we demonstrate that liver tumours testing HBV DNA positive usually present integrated viral sequences thus providing further indication of a pathogenetic role played by the persisting presence of viral sequences in the hepatocytes in terms of tumorigenesis. There is much evidence that patients with overt HBV infection have a high risk of HCC development whereas prospective studies evaluating the incidence of HCC occurrence in OBI carriers are still lacking. However, in many areas of the world, including the most developed countries, HCC is reported to very frequently occur in patients with chronic HCV infection. Now, OBI may contribute to HCC development in HCV patients, as above mentioned. Thus, it seems confirmed that HBV is a risk factor of HCC development also in OBI individuals, and the integration event might play a major role.

Occult HBV infection is a well-known danger for human health in terms of risk of viral reactivation in conditions of immunosuppression as well as of transmission of the infection during liver transplantation. The present study may represent a further evidence that OBI exerts a pro-oncogenic role and its identification in patients with chronic hepatitis or cirrhosis may make it possible to identify individuals at high risk of cancer development. Considering that diagnosis of OBI currently relies on non-standardized techniques and can be performed only in highly specialized laboratories, we do hope that valid and commercially available assays will be developed in the very near future in order to allow the detection of OBI in all cases in which its presence might represent a clinical risk.

References

- 1 El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; 142: 1264–73 e1.
- 2 Pollicino T, Saitta C, Raimondo G. Hepatocellular carcinoma: the point of view of the hepatitis B virus. *Carcinogenesis* 2011; 32: 1122–32.
- 3 Tsai WL, Chung RT. Viral hepatocarcinogenesis. *Oncogene* 2010; 29: 2309–24.
- 4 Raimondo G, Allain JP, Brunetto MR, et al. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol* 2008; 49: 652–7.
- 5 Korba BE, Wells FV, Baldwin B, et al. Hepatocellular carcinoma in woodchuck hepatitis virus-infected woodchucks: presence of viral DNA in tumor tissue from chronic carriers and animals serologically recovered from acute infections. *Hepatology* 1989; 9: 461–70.
- 6 Pollicino T, Squadrito G, Cerenzia G, et al. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology* 2004; 126: 102–10.
- 7 Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. Studies in percutaneous liver biopsies and post-mortem tissue specimens. *N Engl J Med* 1981; 305: 1067–73.
- 8 Urashima T, Saigo K, Kobayashi S, et al. Identification of hepatitis B virus integration in hepatitis C virus-infected hepatocellular carcinoma tissues. *J Hepatol* 1997; 26: 771–8.
- 9 Gozuacik D, Murakami Y, Saigo K, et al. Identification of human cancer-related genes by naturally occurring Hepatitis B Virus DNA tagging. *Oncogene* 2001; 20: 6233–40.
- 10 Murakami Y, Saigo K, Takashima H, et al. Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. *Gut* 2005; 54: 1162–8.

- 11 Raimondo G, Pollicino T, Cacciola I, Squadrito G. Occult hepatitis B virus infection. *J Hepatol* 2007; 46: 160–70.
- 12 Minami M, Poussin K, Brechot C, Paterlini P. A novel PCR technique using Alu-specific primers to identify unknown flanking sequences from the human genome. *Genomics* 1995; 29: 403–8.
- 13 Pollicino T, Vegetti A, Saitta C, et al. Hepatitis B virus DNA integration in tumour tissue of a non-cirrhotic HFE-haemochromatosis patient with hepatocellular carcinoma. *J Hepatol* 2013; 58: 190–3.
- 14 Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature* 1979; 281: 646–50.
- 15 Li J, Lu Y, Zhang J, et al. PI4KIIalpha is a novel regulator of tumor growth by its action on angiogenesis and HIF-1alpha regulation. *Oncogene* 2010; 29: 2550–9.
- 16 Purdue MP, Johansson M, Zelenika D, et al. Genome-wide association study of renal cell carcinoma identifies two susceptibility loci on 2p21 and 11q13.3. *Nat Genet* 2011; 43: 60–5.
- 17 Di Santo R, Maga G. Human terminal deoxynucleotidyl transferases as novel targets for anticancer chemotherapy. *Curr Med Chem* 2006; 13: 2353–68.
- 18 Tong WG, Wierda WG, Lin E, et al. Genome-wide DNA methylation profiling of chronic lymphocytic leukemia allows identification of epigenetically repressed molecular pathways with clinical impact. *Epigenetics* 2010; 5: 499–508.
- 19 Dorn L, Menezes LF, Mikuz G, et al. Immunohistochemical detection of polyductin and co-localization with liver progenitor cell markers during normal and abnormal development of the intrahepatic biliary system and in adult hepatobiliary carcinomas. *J Cell Mol Med* 2009; 13: 1279–90.
- 20 Kan Z, Jaiswal BS, Stinson J, et al. Diverse somatic mutation patterns and pathway alterations in human cancers. *Nature* 2010; 466: 869–73.
- 21 Garcia-Marcos M, Ghosh P, Farquhar MG. Molecular basis of a novel oncogenic mutation in GNAO1. *Oncogene* 2011; 30: 2691–6.
- 22 Homig-Holzel C, van Doorn R, Vogel C, et al. Antagonistic TSC22D1 variants control BRAF(E600)-induced senescence. *EMBO J* 2011; 30: 1753–65.
- 23 Bonilla Guerrero R, Roberts LR. The role of hepatitis B virus integrations in the pathogenesis of human hepatocellular carcinoma. *J Hepatol* 2005; 42: 760–77.
- 24 Yaginuma K, Kobayashi H, Kobayashi M, et al. Multiple integration site of hepatitis B virus DNA in hepatocellular carcinoma and chronic active hepatitis tissues from children. *J Virol* 1987; 61: 1808–13.
- 25 Minami M, Daimon Y, Mori K, et al. Hepatitis B virus-related insertional mutagenesis in chronic hepatitis B patients as an early drastic genetic change leading to hepatocarcinogenesis. *Oncogene* 2005; 24: 4340–8.
- 26 Jiang Z, Jhunjhunwala S, Liu J, et al. The effects of hepatitis B virus integration into the genomes of hepatocellular carcinoma patients. *Genome Res* 2012; 22: 593–601.

- 27 Sung WK, Zheng H, Li S, et al. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. *Nat Genet* 2012; 44: 765–9.
- 28 Fujimoto A, Totoki Y, Abe T, et al. Whole-genome sequencing of liver cancers identifies etiological influences on mutation patterns and recurrent mutations in chromatin regulators. *Nat Genet* 2012; 44: 760–4.
- 29 Bréchet C, Hadchouel M, Scotto J, et al. State of hepatitis B virus DNA in hepatocytes of patients with hepatitis B surface antigen-positive and -negative liver diseases. *Proc Natl Acad Sci USA* 1981; 78: 3906–10.
- 30 European Association for the Study of The Liver. EASL clinical practice guidelines: management of chronic hepatitis B virus infection. *J Hepatol* 2012; 57: 167–85.
- 31 Cha C, Dematteo RP. Molecular mechanisms in hepatocellular carcinoma development. *Best Pract Res Clin Gastroenterol* 2005; 19: 25–37.
- 32 Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002; 31: 339–46.
- 33 Lara-Pezzi E, Majano PL, Gomez-Gonzalo M, et al. The hepatitis B virus X protein up-regulates tumor necrosis factor alpha gene expression in hepatocytes. *Hepatology* 1998; 28: 1013–21.
- 34 Cougot D, Neuveut C, Buendia MA. HBV induced carcinogenesis. *J Clin Virol* 2005; 34(Suppl 1): S75–8.
- 35 Chemin I, Zoulim F. Hepatitis B virus induced hepatocellular carcinoma. *Cancer Lett* 2009; 286: 52–9.
- 36 Squadrito G, Cacciola I, Alibrandi A, Pollicino T, Raimondo G. Impact of occult hepatitis B virus infection on the outcome of chronic hepatitis C. *J Hepatol* 2013; 59: 696–700.